Journal of Medical Virology 61:221–227 (2000)

Evaluation of Enterovirus Serological Tests IgM-EIA and Complement Fixation in Patients With Meningitis, Confirmed by Detection of Enteroviral RNA by RT-PCR in Cerebrospinal Fluid

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An enzyme immunoassay (EIA) for detection of anti-enterovirus IgM antibodies was compared with complement fixation test in 43 patients with confirmed enterovirus meningitis by RT-PCR of cerebrospinal fluids (CSF). In 34% of patients with enterovirus meningitis, IgM antibodies could be found, whereas complement fixation tests were positive in only 20%. The specificity was determined with sera of 105 patients with non-enterovirus meningitis. Specificity of IgM EIA and of complement fixation was 94% and 85%, respectively. In four patients with meningitis but without enterovirus detection in CSF, RT-PCR and virus isolation from stools were positive. In three of these patients, IgM antibodies were detected, giving a strong indication of an enterovirus-associated disease. Because of the high specificity of IgM EIA, diagnosis of enterovirus-associated diseases can be carried out in a single serum sample, whereas by complement fixation tests, only fourfold increases in antibody titres in paired sera indicate an acute infection. The application of IgM EIA is especially important in cases of meningitis when CSF samples are not available and for diagnosis of enterovirus diseases with other clinical symptoms such as fever, enteritis, and hand-foot-and-mouth disease. ***J. Med. Virol. 61:221–227, 2000.***

KEY WORDS: enterovirus; coxsackievirus; ECHO-virus; enzyme immunoassay; IgM; complement fixation; PCR; virus isolation; meningitis

INTRODUCTION

Enteroviruses are the most common cause of aseptic meningitis. Virological diagnosis is important to distinguish between enterovirus-induced meningitis or encephalitis and other treatable bacterial and viral diseases. Furthermore, the discovery of Pleconaril, an orally active broad-spectrum antipicornaviral agent, opens perspectives for special target treatment of enterovirus infections [Abdel-Rahman and Kearns, 1998].

Laboratory diagnosis of enterovirus infections is based on detection of virus in clinical specimens. The standard procedure for detection of enteroviruses is virus isolation in cell culture. This method is labour-intensive and time-consuming. More rapid shell vial cultures lack sensitivity in comparison to conventional virus isolation [Van Doornum and De Jong, 1998]. As an alternative rapid test, reverse transcription-polymerase chain reaction (RT-PCR) can be used for routine enterovirus diagnosis. RT-PCR is more sensitive than cell culture methods because of detection of infectious and noninfectious virus particles and results can be obtained within one day [Searle et al., 1997; Gorgievski-Hrisoho et al., 1998]. RT-PCR is also useful for detection of enterovirus serotypes that cannot be propagated in cell culture. However, the PCR technique requires special equipment and staff. A new rapid and reliable enzyme immunoassay (EIA) for detection of enterovirus antigen, which only requires standard EIA laboratory equipment, was recently developed [Terletskaia-Ladwig et al., in press].

The examination of specific enteroviral antibody response is an alternative approach for diagnosis. The availability of different serological tests for the diagnosis of enterovirus infections was investigated intensively in 1990–1995 [Frisk et al., 1989; Enders et al., 1990; Muir and Bantalava, 1990; Boman et al., 1992; Swanink et al., 1993; Samuelson et al., 1993; Hodgson et al., 1995]. Enzyme immunosorbent assays were found to be simple to carry out, more sensitive than the

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Accepted 26 November 1999

complement fixation test, and far less laborious than the neutralisation test. It was demonstrated that the detection of IgM-specific antibodies permits rapid confirmation of acute enterovirus infections, whereas IgA antibodies are less suitable. The detection of IgG antibodies can be interpreted only in paired acute and convalescent sera by determination of an at least fourfold increase in antibody titres. A single sample is not useful because of high titres of anti-enterovirus antibodies in healthy individuals. The same applies to the complement fixation test. By contrast, the IgG EIA was shown to be a valid alternative to complement fixation test because of its sensitivity. In all these studies patients were defined as positive for an enterovirus-associated disease on the basis of results of virus isolation in cell culture or complement fixation test because the RT-PCR diagnosis of enteroviruses at that time had not yet been developed. The aim of this study was to show the diagnostic potential of enteroviral serological tests by comparison of complement fixation and IgM EIA with results obtained by RT-PCR.

MATERIALS AND METHODS

Clinical Samples

Clinical specimens (stool, cerebrospinal fluid [CSF], and sera) were obtained from practitioners and paediatric hospitals in Germany and tested as part of the daily diagnostic routine. They originated from 164 patients suspected to have an enterovirus infection based on symptoms like meningitis, encephalitis, hand-foot-mouth-disease, or gastrointestinal symptoms, and for whom in part information concerning the onset of illness was available. Most samples were received from children younger that 10 years. Sera for serological tests and at least one sample CSF or stool for detection of viruses were available from all patients.

For definition of the cut-off level for the serological tests, 50 sera from healthy pregnant women and 50 healthy children (sera were sent to the laboratory for examination of immune status before vaccination) and from 100 healthy blood donors were used.

Virus Isolation

In this study, 0.5–1 g stool was suspended in 5 ml phosphate-buffered saline (PBS) and centrifuged for 5 min at 1,200*g* for virus isolation. The supernatant was sterile filtered (0.2 μm), and 200 μl each of the suspension was inoculated in duplicate onto human embryonic lung fibroblasts (HEL), primary monkey kidney cells (PMK), and human colonic carcinoma cells (CaCo-2). Before testing, CaCo-2 cells were preincubated overnight in minimal essential medium with Earle's salts and nonessential amino acids with 4 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma Chemical Co., St. Louis, MO) and without foetal calf serum (FCS) in order to activate virus at inoculation [Pinto et al., 1994]. Maintenance medium for CaCo-2 cells (E-MEM with antibiotics) also contained 4 μg/ml TPCK-treated trypsin. All tissue cultures were incubated at 37°C for up to 10 days and checked daily for cytopathic effects (CPE). The enteroviral CPE was confirmed by immunofluorescence antibody (IFA) staining using the enterovirus screening set (Chemicon, Temecula, CA).

Isolation of Viral Nucleic Acid

Viral nucleic acid was extracted from CSF with the High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Stool samples were suspended in PBS, centrifuged and 200 μl of supernatant were used for nucleic acid isolation as described above. Suitable positive and negative controls were extracted along with the clinical samples and were included in every run.

Primers and RT-PCR

Primers and PCR conditions are described elsewhere [Searle et al., 1997]. Briefly, primers for cDNA synthesis and PCR were designed to amplify a part of the highly conserved 5′ noncoding region of the enteroviral genome. cDNA synthesis and PCR were performed in 50 μl reaction volumes using the Expand® Reverse Transcriptase for RT and Taq-DNA-Polymerase for PCR (Roche Diagnostics GmbH, Mannheim, Germany).

Detection of PCR Products

Detection of PCR products was carried out using the commercial PCR-ELISA-System (Roche Diagnostics GmbH, Mannheim Germany), according to the manufacturer's instruction with slight modifications. All steps, including DIG detection, washing, substrate incubation, and photometric measurement, were done at 37° C in a Behring ELISA-Processor III (Behringwerke, Marburg, Germany). Precautions were taken to avoid false-positive PCR results during nucleic acid isolation and subsequent PCR steps [Kwok and Higuchi, 1989].

Sensitivity of RT-PCR

The sensitivity of the RT-PCR was estimated in comparison with isolation of virus in cell culture [Searle et al., 1997]. Virus stocks (coxsackievirus B5 and coxsackievirus A16) were diluted 10-fold up to a dilution of 10^{-12}. The infectivity of the virus stocks was measured by determining the 50% tissue culture infective dose ($TCID_{50}$/ml) in a microtitre plate assay. The RT-PCR detected 1.9 $TCID_{50}$ of coxsackievirus B5 and 0.06 $TCID_{50}$ coxsackievirus A16, respectively.

Serotype Specificity of RT-PCR

The suitability of the primers to amplify different enterovirus types was established with a panel of viruses: coxsackieviruses A5, A9, A10, A16, B1, B2, B3, B4, and B5; echoviruses 4, 9, 11, 25, and 30; and polioviruses 1, 2, and 3 [Searle et al., 1997]. The RT-PCR detected all 18 types of enteroviruses.

EIA for Detection of Anti-enterovirus IgM, IgA, IgG Antibodies

Microtitre plates (Polysorp, Nunc, Denmark) were coated overnight at 4°C with purified Coxsackievirus B5 and ECHO9-virus, propagated in Vero cells, and with control antigen derived from noninfected Vero cells. Sera for testing of IgM antibodies were pretreated with rheumatoid factor (RF) absorbent (Behringwerke, Marburg, Germany), diluted 1:100 in PBS with 0.05% Tween 20 supplemented with 1% bovine serum albumin and applied in 100 μl aliquots to coated wells. Plates were incubated for 1 hr at 37°C. After washing three times with PBS with 0.05% Tween 20, corresponding to test rabbit anti-human IgM, IgA, IgG horseradish peroxidase (HRP) conjugates (Behringwerke) were added to each well. After incubation at 37°C for 30 min, plates were washed four times, and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added. The reaction was stopped with 50 μl 1 M sulfonic acid and A_{450} was measured. The titre was determined as difference between absorbency of antigen and control antigen. Results were considered positive or negative with respect to cut-off level. The cut-off was defined as the duplicate mean absorbence of 200 serum specimens from healthy persons.

Complement Fixation Test

The complement fixation (CF) test was carried out with picornavirus antigen (Virion, Planegg, Germany). Only single acute sera were tested. Therefore borderline levels of antibody titres has been defined (see also Table V). A titre of <64 was considered negative, 1:128 borderline, and >1:256 positive.

Detection of Autoantibodies anti-SSA (Ro) and Anti-SSB (La)

Screening for antinuclear antibodies (ANA) was carried out using indirect immunfluorescence on Hep2-cells (Euroimmun, Luebeck, Germany). A titre of 1:320 and higher was considered positive. A commercial EIA (Euroimmun, Luebeck, Germany), based on the affinity-purified single Ro and La antigens, was used for the identification of anti-SSA (Ro) and anti-SSB (La) specificity of ANA in positive sera. Positive EIA results were confirmed by an immunoblot test (Viramed, Planegg/Steinkirchen, Germany).

RESULTS

Sera from 200 healthy persons were tested for definition of the cut-off level of the IgM-EIA. When the cut-off level was set at the duplicate mean absorbence of all tested sera, 95% of sera were negative, 1% was positive and 4% showed borderline titres. By examination of these sera in complement fixation test 67% of sera showed titres of <1:32; 24% 1:64; 6% 1:128, and 3% >1:256; therefore, the cut-off level for the complement fixation test was defined at a titre of 1:128.

Comparison of results of enterovirus detection by both RT-PCR and virus isolation by cell culture on the one hand and detection of antibody response by complement fixation test and IgM EIA on the other was done for 164 patients. In 43 cases, enteroviruses were found by RT-PCR in CSF; in 16 cases, enteroviral RNA was detected by RT-PCR in stool samples. RT-PCR in CSF of 105 patients with meningitis were negative. Virus isolation was done mainly with stool samples. ECHOviruses 11 and 30 were isolated most frequently. Other isolated virus types were ECHOviruses 4, 9, 21, 29, and 71, and Coxsackieviruses A9, B2, and B5. In several cases, no subtyping of virus was carried out, as only a group-specific monoclonal antibody was used (Pan-Enterovirus antibody, 9D5, Chemicon, Temecula, CA) for IFA confirmation.

Table I shows the results of serological tests in 43 patients suffering from enterovirus meningitis confirmed by detection of viral RNA by RT-PCR in CSF. In three of these patients, CSF was also examined by inoculation in cell culture with positive results (cases 1, 11, 23). In 20 cases, stool samples were also available; 18 of these samples were positive by either virus isolation or RT-PCR or both methods. IgM antibodies in serum were detected in 15 (35%) patients with confirmed enterovirus disease by RT-PCR of CSF (cases 1–15), whereas complement fixation test was positive (≥1:256) only in 9 (21%) of these cases. Borderline titres were found 6 times with IgM EIA and also 6 times with complement fixation test (14% each). IgM was not found in 22 cases and by complement fixation antibodies in 28 cases.

Test results of 11 patients with virus detected in stools by either RT-PCR and/or cell culture isolation are shown in Table II. In these cases, only faecal and serum samples were available for examination. Patients suffered from meningitis (5×), enteritis (1×), fever (4×), hand-foot-mouth-disease (1×). In five cases (45%) also IgM antibodies in serum probes could be found. Borderline titres were not determined. Complement fixation was negative in seven cases, borderline titres in three cases and one positive result (10%).

Table III shows results from four patients suffering from meningitis in whom CSF were negative by enterovirus RT-PCR. PCR for other infectious agents causing meningitis such as Herpesviruses or *Borrelia burgdorferi* was negative in all four patients. In all these patients, Enteroviruses were detected in stool samples, and three had positive IgM in serum. Complement fixation was positive in one case. Comparison of sensitivity and specificity of both complement fixation test and EIA is shown in Table IV. The sensitivity of complement fixation test and IgM EIA was calculated by using sera from patients with enterovirus detected in CSF by RT-PCR (total 43). For calculation of specificity, sera from patients with enterovirus negative CSF by RT-PCR (total 105) were used. The sensitivity of complement fixation test was 21% (9/43) and of IgM EIA 35% (15/43). The specificity of complement fixation test was 85% (90/105) and of IgM EIA 94% (99/105).

Results of complement fixation tests in 105 patients with negative RT-PCR in CSF were positive: >1:256 in

TABLE I. Results of IgM EIA and Complement Fixation Test from Patients With Confirmed Enterovirus Meningitis by RT-PCR in Cerebrospinal Fluid

Patient no.	Days after onset	Age	RT-PCR CSF	Cell culture CSF	RT-PCR stool	Cell culture stool	Complement fixation (1:X)	IgM EIA
1	4	6 yr	+	+CVA9	nd	nd	256	+
2	4	7 yr	+	nd	nd	nd	256	+
3	2	8 yr	+	nd	nd	EV11	256	+
4	3	7 yr	+	nd	+	EV11	128	+
5	3	6 yr	+	nd	+	EV	128	+
6	17	1 yr	+	nd	+	–	128	+
7	4	6 yr	+	nd	nd	nd	128	+
8	4	7 yr	+	nd	–	–	64	+
9	?	5 yr	+	nd	nd	nd	64	+
10	5	8 yr	+	nd	nd	nd	64	+
11	4	6 yr	+	+EV11	nd	nd	32	+
12	3	5 yr	+	–	+	–	16	+
13	2	8 yr	+	nd	nd	nd	16	+
14	?	7 yr	+	nd	nd	nd	16	+
15	3	4 yr	+	nd	+	EV11	8	+
16	?	7 yr	+	nd	nd	nd	256	+/–
17	3	6 yr	+	nd	+	CVB	128	+/–
18	2	6 yr	+	nd	nd	nd	64	+/–
19	3	10 yr	+	nd	nd	nd	32	+/–
20	?	10 yr	+	nd	nd	nd	32	+/–
21	3	13 yr	+	nd	+	EV4	8	+/–
22	3	5 yr	+	–	+	–	512	–
23	2	8 yr	+	+EV30	nd	nd	256	–
24	?	33 yr	+	nd	+	nd	256	–
25	1	9 yr	+	nd	nd	nd	256	–
26	1	4 yr	+	nd	nd	nd	256	–
27	2	6 yr	+	nd	nd	nd	128	–
28	7	10 yr	+	nd	+	EV9	64	–
29	3	6 yr	+	nd	nd	EV11	64	–
30	?	14 yr	+	nd	nd	nd	64	–
31	2	11 yr	+	nd	nd	nd	64	–
32	3	5 yr	+	nd	nd	nd	64	–
33	3	12 yr	+	nd	nd	nd	64	–
34	3	13 yr	+	nd	+	EV30	32	–
35	4	13 yr	+	nd	nd	nd	32	–
36	2	4 yr	+	nd	+	CVB	32	–
37	2	2 yr	+	nd	–	–	16	–
38	5	5 yr	+	nd	nd	nd	16	–
39	?	6 yr	+	nd	nd	nd	16	–
40	2	4 yr	+	nd	+	EV4	8	–
41	2	7 yr	+	nd	+	EV30	8	–
42	2	6 yr	+	–	+	EV	8	–
43	2	2 wk	+	nd	+	nd	8	–

nd, not determined, yr, years; wk, weeks; CSF, cerebrospinal fluid; +/–, grey zone; CVA, Coxsackievirus A, CVB, Coxsackievirus B; EV, enterovirus; RT-PCR, reverse transcription-polymerase chain reaction.

6 (6%) of all cases; borderline: 1:128 in 9 (9%), negative: 1:64 in 23 (22%), and <1:32 in 67 (63%). These data confirm the correct definition of the cut-off level at a titre of 1:128 for the complement fixation test. If the cut-off level for this test would be defined at a titre of 1:64 the specificity of complement fixation would be only 63%.

One serum from a patient with negative RT-PCR result of CSF showed a high titre of 1:4096 in complement fixation test. This patient, a 6-year-old child, had one onset of spasms. Autoantibodies against Ro (SS-A) and La (SS-B) antigens in the serum of this child could be detected. Testing of 26 anti-Ro and -La positive sera with titre of antinuclear antibodies >1:320 demonstrated titres of 1:256–1:4096 by complement fixation test with picornavirus antigen in 9 sera (34%) (Table V), one serum had a titre of 1:128, and others were negative. To exclude any nonspecific reaction with cellular antigens of Vero cells, which were used for propagation of enteroviruses, sera were also tested in respiratory syncytial virus (RSV) antigen by complement fixation test, because RSV is propagated in the same Vero cell line as enteroviruses. All sera were negative in RSV complement fixation test. All these sera were also negative for anti-enteroviral IgM-antibodies and no correlation was found between complement fixation and anti-enteroviral IgG and IgA antibody titres.

DISCUSSION

Development of RT-PCR, the most sensitive and also very rapid test for the diagnosis of enteroviral infections, led to the opinion, that other tests are unnecessary. Most recent reports describe the diagnostic advantages of RT-PCR for CSF [Tanel et al., 1996;

TABLE II. Results of Serological Tests in IgM EIA and Complement Fixation Test from Patients With Positive Enterovirus Detection by RT-PCR in Stools

Patient no.	Days after onset	Age	Clinical date	RT-PCR stool	Cell culture stool	Complement fixation (1:x)	IgM EIA
1	10	10 yr	Meningitis	+	EV	256	+
2	15	34 yr	Headache, fever	+	EV30	128	+
3	5	41 yr	Headache, fever	+	EV30	64	+
4	8	1 yr	Headache, fever	+	EV30	32	+
5	2	12 yr	Meningitis	+	EV11	16	+
6	2	14 yr	Meningitis	+	EV	128	–
7	28	21 yr	Headache, fever	+	–	128	–
8	8	26 yr	Head-foot-and-mouth disease	+	–	64	–
9	3	6 yr	AV block	+	EV30	32	–
10	3	12 yr	Meningitis	+	–	32	–
11	3	2 wk	Enteritis	+	CVB5	16	–

yr, years; wk, weeks; CSF, cerebrospinal fluid; CVB, Coxsackievirus B; EV, enterovirus; RT-PCR, reverse transcription-polymerase chain reaction.

TABLE III. Results of Serological Tests in IgM EIA and Complement Fixation Test from Four Patients With Meningitis and Positive Enterovirus Detection by RT-PCR and Virus Isolation in Stools and Negative RT-PCR in CSF

Patient no.	Days after onset	Age	RT-PCR CSF	Cell culture CSF	RT-PCR stool	Cell culture stool	Complement fixation (1:X)	IgM EIA
1	3	8 yr	–	–	+	EV	256	+
2	9	4 yr	–	nd	+	EV	128	+
3	3	3 yr	–	nd	+	EV70/71	8	+
4	4	2 yr	–	nd	+	EV9	64	–

nd, not determined; yr, years; CSF, cerebrospinal fluid; EV, enterovirus; RT-PCR, reverse transcription-polymerase chain reaction.

TABLE IV. Sensitivity and Specificity of IgM EIA and Complement Fixation

Patients	No. of samples	% sensitivity[a]	% specificity[b]
Enteroviral RT-PCR-positive CSF			
Total 43			
Complement fixation		20 (9/43)	
Positive ≥256	9		
Borderline = 128	6		
Negative ≤64	28		
IgM EIA		34 (15/43)	
Positive ≥1.1	15		
Borderline 0.9–1.1	6		
Negative ≤0.9	22		
Enterovirual RT-PCR-negative CSF			
Total 105			
Complement fixation			85 (90/105)
Positive ≥256	6		
Borderline = 128	9		
Negative ≤64	90		
IgM EIA			94 (99/105)
Positive ≥1.1	1		
Borderline 0.9–1.1	5		
Negative ≤0.9	99		

*Comparison of results from patients with positive and negative RT-PCR in CSF.
[a]Number of true positives/total number of patients with RT-PCR positive in CSF.
[b]Number of true negatives/total number of patients with RT-PCR negative in CSF.

Marshall et al., 1996; van Viet et al., 1998; Furione et al., 1998]. But in laboratories for routine clinical diagnosis the most common specimens for diagnosis of enterovirus-associated diseases are serum samples sent by practitioners and paediatricians, whereas CSF samples are only taken in hospitals.

The usefulness of faecal samples for enterovirus diagnosis in patients with aseptic meningitis is shown in the present study and was also shown by Glimaker et al. [1992]. However, only virus detection in CSF or vesicle fluid is etiological evidence of an enterovirus-associated disease. In this study, etiological evidence of

TABLE V. Comparison of Serological Tests Complement Fixation, Enterovirus IgM, IgA, and IgG EIA in Sera from Patients With Autoantibodies Against Ro (SS-A) and La (SS-B) Antigens

Patient no.	Complement fixation with enterovirus antigen	Antinuclear antibodies (immunofluorescence)	Enterovirus IgM	Enterovirus IgA	Enterovirus IgG	Complement fixation with RSV antigen
1	1:4,096	1:5,120	–	+/–	–	1:32
2	1:4,096	1:640	–	–	–	1:16
3	1:4,096	1:2,560	–	–	–	<1:16
4	1:2,048	1:1,280	–	–	+/–	1:16
5	1:1,024	1:640	–	–	–	1:16
6	1:256	1:1,280	–	+	+/–	1:64
7	1:256	1:5,120	–	+/–		+
8	1:256	1:1,280	–	–	–	<1:16
9	1:256	1:5,120	–	–	–	1:32

+/–, grey zone; RSV, respiratory syncytial virus.

an enterovirus-associated meningitis was confirmed by RT-PCR of CSF. Virus isolation from CSF was carried out only a few cases, as most specimens originated from children (Tables I, II); therefore, only small quantities were available. Thus RT-PCR was defined as standard.

On the other hand, RT-PCR results can be negative in CSF in patients with meningitis, although virus in stool is detectable. Gorgievski-Hrisoho et al. [1998] found such discrepant results in three cases. In the present study, enteroviral genomes were detected in stool from four patients who had enterovirus-negative CSF by RT-PCR (Table III; see also below). If the results of RT-PCR in stool and CSF are discrepant, or only one of these specimens is available, alternative serological methods are very helpful. In addition, sera are useful specimens for differential diagnosis, which is important for suspected enteroviral infection, because these viruses cause very different clinical symptoms, which are also caused by other infectious agents. Although the value of EIA in enterovirus serology is well documented [Enders et al., 1990; Muir and Bantalava, 1990; Boman et al., 1992; Samuelson et al., 1993; Swanink et al., 1993], most laboratories do not provide any serological enterovirus diagnosis at all or only use the complement fixation test. This situation is partially related to the limited availability of EIA test kits on the market.

For the development of the enterovirus IgM EIA in this study, Coxsackievirus B5 and ECHO-virus 9 (EV9) were used. The cross-reactivity of these viruses was sufficient for the detection of IgM antibodies in patients infected with 10 different enterovirus types. Boman et al. [1992] described an EIA based on Coxsackie B5 antigen, that was positive in patients with 12 different enterovirus serotypes. The cross-reactivity between different serotypes of the genus enterovirus can be explained by the existence of a group-specific antigenic determinant [Klespies et al., 1996]. Therefore, it is possible to use only two types of enteroviruses for the production of an EIA with broad type specificity.

The complement fixation method and IgM EIA were evaluated using sera from patients with known results of RT-PCR of CSF. The sensitivity of IgM EIA (34%) was higher than that of complement fixation test (20%). Although the sensitivity of IgM EIA is not very high, the specificity of this test is >95%, as shown by the results of sera screening in healthy persons and >90% by the results of sera examination from patients without any confirmation of enteroviral infection by RT-PCR. By contrast, the complement fixation test is of low sensitivity and is also less specific; 9% of healthy persons and 14% of enterovirus RT-PCR-negative persons had complement fixation antibody titres >1:128 (Table V). Furthermore, the surprisingly high and frequent anti-enterovirus complement fixation antibody titres in patients with anti-Ro and anti-La antibodies were not associated with increased IgG, IgA, or IgM antibodies and, therefore, do not confirm an acute enteroviral infection. However, the high titre of complement fixation antibodies may indicate an enteroviral relation to the pathophysiology of autoimmune diseases. Possibly, in the complement fixation test, other subclasses of antibodies are involved in immunological reactions, than by EIA. The antinuclear autoimmune antibodies in complement fixation test may cross-react with enteroviral antigen. The role of enteroviruses in the development of autoimmune diseases is being researched intensively. The prevalence of Coxsackie B virus complement fixation antibodies in patients with juvenile dermatomyositis has been reported [Christensen et al., 1986]. Coxsackie virus infections are discussed as a possible cause of dermatomyositis [Kümmerle-Deschner et al., 1997]. Wekerle [1998] and Kraemer et al. [1998] supposed enteroviral triggering of autoimmune insulin-dependent diabetes mellitus and Graves disease.

Comparison of the sensitivity of complement fixation test with IgM EIA in the group of patients in whom only stool samples were examined by RT-PCR also showed the advantage of the IgM EIA (Table II). The sensitivity of IgM EIA is 45% and that of complement fixation test only 10%. The application of IgM EIA together with virus detection by cell culture allows the diagnosis of enterovirus-associated diseases with other clinical symptoms such as fever, enteritis, and hand-foot-and-mouth disease. Furthermore, in the present study, enteroviral genomes were detected in stool probes from four patients, who had enterovirus negative CSF by RT-PCR (Table III). In three of these cases, IgM antibodies in serum samples were detected, providing strong evidence of an enterovirus-associated meningitis.

In contrast to the complement fixation test, the enterovirus IgM EIA allows rapid and reliable diagnosis using a single serum sample and has a specificity of about 95% and a sensitivity of 34%.The low sensitivity can be explained by the fact that an IgM antibody response will not develop in all cases of enterovirus-associated disease. Looking at the data in Tables I and II, IgM antibodies were detectable in a wide range of 2–17 days after onset of symptoms. Swanink et al. [1993] reported that the sensitivity of the IgM EIA increased if the serum was collected later than 3 days after the onset of symptoms, and in patients older than 1 year. The appearence of enteroviral IgM is usually transient, but occasionally IgM was found to persist for 1 year [Samuelson et al., 1990]. The prevalence of enteroviral IgM in healthy persons is lower than 3%. The complement fixation test can be used for diagnosis only with paired acute and convalescent serum samples by demonstrating a fourfold or greater increase of antibodies. Thus, this test is unsuitable for rapid diagnosis.

In conclusion, the data demonstrate the need and usefulness of alternative methods for enterovirus diagnosis. The complementary use of different samples, namely CSF, stool and serum samples, and alternative methods such as RT-PCR, virus isolation, and IgM antibody detection, improve the diagnosis of enterovirus-associated disease.

ACKNOWLEDGMENTS

We thank Helmuth Schröder, Michael Leinmüller, Silvia Meier, Sylvia Boddien, and Sabine Wunderlich for excellent technical assistance.

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